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Ribotyping of *Helicobacter pylori* from Clinical Specimens

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Ribotyping is a method used to type strains of bacteria by analyzing the restriction enzyme digestion patterns of the rRNA genes. This method was applied to 126 strains of *Helicobacter pylori* from 100 unrelated symptomatic patients who had endoscopies done and to 15 strains from 15 infected subjects from seven families. Analysis of the rRNA gene patterns revealed 77 distinct ribotypes from the 100 patients. From 15 of these subjects, isolates were recovered from antral mucosal biopsies at follow-up endoscopy. All follow-up isolates from the same patient, with one exception, yielded identical digest patterns. This patient had strains with two distinct digest patterns obtained from a set of three isolates cultured from biopsy specimens taken at different times. Five patients who had isolates recovered from different sites in the stomach (antrum, gastric body, duodenum, and pyloric channel) showed ribotyping patterns which were identical for each patient yet distinct between patients. In seven family groups studied, identical digest patterns were detected in members of two families, with variability in strains detected among members of the remaining families. This study demonstrates that ribotyping provides a useful, reliable, reproducible, and highly discriminatory typing scheme for the study of *H. pylori* infection.

Helicobacter pylori, first recovered from gastric biopsies by Warren (24) and Marshall (14) almost a decade ago, has now been identified worldwide as a common infection involving the upper gastrointestinal tract. This organism is the cause of type B gastritis (15, 21), plays a significant pathogenic role in duodenal ulcer disease in humans (7, 9, 10, 12), and may yet prove to be of importance in the etiology of some gastric cancers (19, 20). However, little is known about the natural history, pathogenic mechanisms, and mode(s) of transmission of this organism. The source of infection has not been established so far, although it is most likely spread from human to human. The paucity of knowledge in these areas is in part due to the lack of a reliable universal typing method. Classical typing schemes for differentiating bacterial strains within species, such as biotyping, serotyping, and phage typing, appear to be unavailable for this organism. Other alternative typing methods, such as immunoblot fingerprinting (4), plasmid profile typing (6, 23), and restriction endonuclease analysis of chromosomal DNA (11, 23), have been used recently to discriminate strains of *H. pylori*. rRNA gene restriction patterns have been used successfully to differentiate organisms such as coagulase-negative staphylococci (3) and *Salmonella typhi* (1) and recently in a small-sample study of *Helicobacter* spp. (18). In this study, we have extended the sample population and applied the technique in a clinical setting by investigating 100 symptomatic unrelated patients and seven families (15 subjects) who were culture positive for *H. pylori*.

MATERIALS AND METHODS

Patients. (i) Part A. One hundred culture-positive patients (excluding the family groups) were enrolled for this study. The clinical diseases in these patients included nonulcer dyspepsia (37 patients, including 2 with gastric erosions and erythema and 10 with duodenal erosions and erythema), duodenal ulcer (37 patients), gastric ulcer (18 patients), esophageal reflux (5 patients), and carcinoma of the stomach (3 patients).

(ii) Part B. Subjects from seven families (15 subjects) were entered into the study. The clinical profile of each subject is shown in Table 1. The subjects in families 1, 2, and 5 were living together at the time of endoscopy. The subjects in family 3 had never lived together, with all other families living together for a minimum of 18 years.

Isolation and identification of *H. pylori*. Gastric mucosal biopsy specimens obtained at routine endoscopy were finely minced with a sterile scalpel blade and then vortexed at high speed in 1 ml of saline in a sterile 0.5-oz (ca. 15 ml) bottle containing glass beads. A drop of tissue suspension was inoculated onto each of the two selective plates (Skirrow's medium and Dent's CP medium; Oxoid, Basingstoke, England) and a chocolate agar. After incubation at 37°C in microaerobic conditions (10% CO_2, 6% O_2, 84% N_2) for 5 to 7 days, suspect colonies that exhibited typical colonial morphology were identified as *H. pylori* if they hydrolyzed urea rapidly (within 10 min at room temperature), produced catalase, were oxidase positive, did not reduce nitrate, and showed characteristic curved gram-negative bacilli upon Gram staining. The isolates were then preserved at −70°C for typing. To maintain the maximum number of strains available for typing, several subcultures (up to four) were performed on each strain and these isolates were also kept at −70°C.

Extraction of chromosomal DNA. Stored isolates of *H. pylori* were regrown on 6% sheep blood agar and incubated for 5 to 7 days at 37°C under microaerobic conditions. Bacterial cells from 10 plates each were suspended in 5-ml portions of TE buffer (Tris-EDTA buffer, pH 7.6). Extraction and purification of chromosomal DNA were performed

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TABLE 1. Clinical profiles of subjects in the seven families studied

Family	Patient no.	Age (yr)	Relationship	Endoscopic finding[a]
1	F1a	27	Son	NUD
	F1b	52	Mother	NUD
	F1c	60	Father	NUD
2	F2a	27	Son	DU
	F2b	55	Father	NUD
3	F3a	72	Grandfather	NUD
	F3b	24	Grandson	DU
4	F4a	43	Mother	NUD
	F4b	18	Son	NUD
5	F5a	48	Brother	GU
	F5b	46	Brother	DU
6	F6a	53	Brother	DU
	F6b	56	Brother	DU
7	F7a	47	Daughter	NUD
	F7b	72	Mother	RO

[a] GU, gastric ulcer; DU, duodenal ulcer; NUD, nonulcer dyspepsia; RO, reflux esophagitis.

as previously described (2). The concentration and quality of DNA samples were estimated after agarose gel electrophoresis with DNA standards.

DNA probe synthesis. Plasmid pKK 3535, kindly provided by Altwegg and colleagues (1), was digested with *Pst*I and labelled with digoxigenin using the nonradioactive DNA Labelling and Detection Kit of Boehringer GmbH (Mannheim, Germany) according to the manufacturer's instructions. This plasmid DNA probe encodes 5S, 16S, and 23S RNA and $tRNA_2^{Glu}$ genes.

Southern blot hybridization. DNA samples (~2 μg) were digested with 30 U (each) of *Hin*dIII or *Hae*III restriction enzyme at 37°C for 16 h under the conditions specified by the manufacturers. DNA fragments were separated by electrophoresis through 0.7% agarose gels (20 cm long) at 55 V for 17 h in 40 mM Tris-acetate–2 mM EDTA. DNA was transferred to nylon filters (GeneScreen Plus; Dupont, Boston, Mass.) using a vacuum transfer apparatus (Hybaid, Middlesex, England) at 40-cm H_2O vacuum for 1 h with 0.5 M NaOH–1.5 M NaCl solution. The filters were removed and baked at 120°C for 30 min. Prehybridization was carried out at 37°C for 2 h in hybridization solution (50% formamide, 1.75% Sarkosyl, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1% sodium dodecyl sulfate [SDS], 200 μg of herring sperm DNA per ml). Heat-denatured DNA probe in hybridization solution was then added, and the filters were incubated at 37°C overnight. After hybridization, the filters were subjected to two 5-min washes at room temperature with 2× SSC–0.1% SDS and two 30-min washes at 50°C with 0.1× SSC–0.1% SDS. The presence of digoxigenin in the labelled DNA probe was detected with an alkaline phosphatase-conjugated antibody as described in the nonradioactive detection kit (Boehringer). However, slight modification was made when 1% casein in 0.1 M Tris (pH 7.5)–0.15 M NaCl was used in the blocking step and as a conjugate diluent.

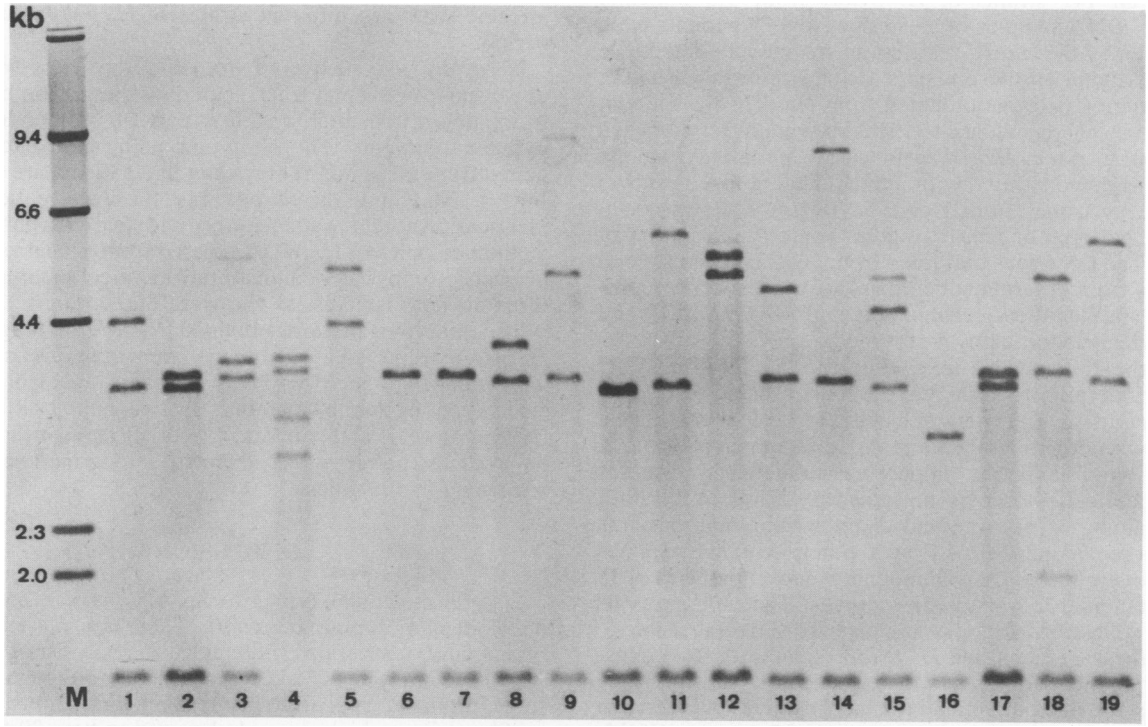

FIG. 1. rRNA gene restriction patterns of *H. pylori* DNA digested with *Hin*dIII from randomly selected patients with various clinical diseases. Lane M, molecular weight markers; lanes 1 to 19, *H. pylori* strains from subjects 1 to 19, respectively. (Subjects 2, 3, 4, 9, 10, 11, 13, 15, and 16 had duodenal ulcers, subjects 1 and 6 had reflux esophagitis, subject 5 had carcinoma of the stomach, and subjects 7, 8, 12, 14, 17, 18, and 19 had nonulcer dyspepsia).

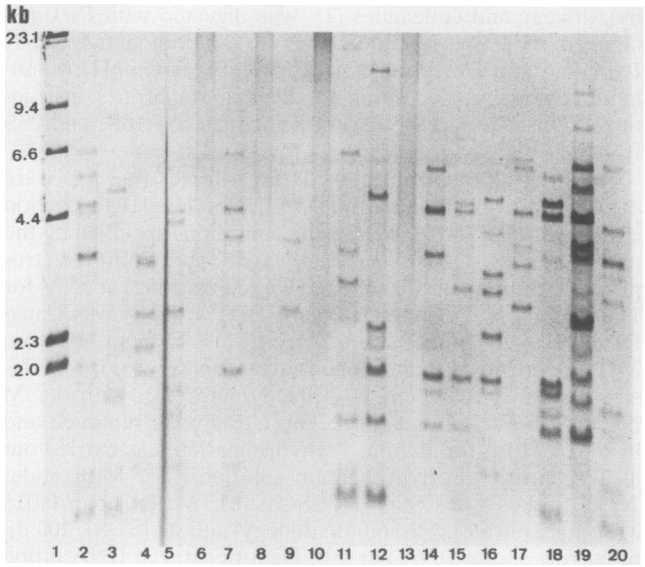

FIG. 2. rRNA gene restriction patterns of *H. pylori* DNA digested with *Hae*III from randomly selected patients with various clinical diseases. Lane 1, molecular weight markers; lanes 2 to 20, *H. pylori* strains from subjects 1 to 19, respectively. (Subjects 2, 3, 4, 9, 10, 11, 13, 15, and 16 had duodenal ulcers, subjects 1 and 6 had reflux esophagitis, subject 5 had carcinoma of the stomach, and subjects 7, 8, 12, 14, 17, 18, and 19 had nonulcer dyspepsia).

RESULTS

Restriction endonucleases *Hin*dIII and *Hae*III were used for digestion of chromosomal DNA of *H. pylori*. *Hin*dIII digested all 141 strains of *H. pylori* from 115 patients. However, DNA samples of 39 strains from 29 patients were not cleaved by the *Hae*III restriction enzyme into detectable fragments. Some of these results are shown in Fig. 1 and 2. The ribotyping patterns obtained from *Hin*dIII digests consists of 2 to 6 bands, while 4 to 10 bands were observed when restriction enzyme *Hae*III was used. Variability among strains was demonstrated with individual patients, as shown in Fig. 1, by using *Hin*dIII digests. Figure 2 shows corresponding patterns of *Hae*III digests using the same set of isolates. The DNA was extracted from 40 isolates on several occasions from different subcultures with consistent, reproducible, and identical results obtained with *Hin*dIII and *Hae*III when ribotyping was repeated.

From the 100 subjects (excluding the family groups), 77 distinct ribotyping patterns were obtained using restriction enzyme *Hin*dIII. Thirty-six subjects shared identical digest patterns with others and were grouped as shown in Table 2. Subjects with different upper gastrointestinal diseases shared identical ribotyping patterns, as shown in Table 2. The remaining 64 patients had distinct ribotyping patterns which were independent of each other. Multiple isolates were recovered from gastroduodenal mucosal biopsies of 15 patients taken at follow-up endoscopies. The time intervals between collection of biopsy samples are shown in Table 3. Nine of these patients had *H. pylori* isolated on two occasions, while five patients had the organism isolated on three occasions and one patient had the bacterium recovered from four consecutive biopsies over a period of 7 months. Of this group of patients, eight had duodenal ulcers, five had gastric ulcers, and two had multiple duodenal erosions. All ulcer patients were treated with histamine-2 receptor blockers, and none had received an antibiotic and/or colloidal bismuth subcitrate (Denol; Brocades Pharma, Delft, Holland) to eradicate *H. pylori* over the study period. Analysis of these strains by ribotyping indicated that all isolates from the same patient yielded identical ribotyping patterns, regardless of the time interval between endoscopies (ranging from two weeks to 7 months), with the exception of one patient. This patient had both duodenal and gastric ulcers identified at endoscopy with *H. pylori* cultured from antral biopsies on 3 occasions. These three isolates yielded two distinct ribotyping patterns, as shown in Fig. 3, lanes 11, 12, and 13. Repeated subculture of the original isolates yielded similar patterns. This result suggests that the subject had been infected with two different strains of *H. pylori* during this period.

TABLE 2. Ribotyping patterns shared by more than one subject

Ribotyping profile[a]	No. of subjects with the same ribotyping profile	Clinical disease[b]
R4	2	GU; duodenal erosions
R5	2	RO; NUD
R13	2	Ca; DU
R20	2	RO; DU
R25	2	DU; DU
R30	2	NUD; GU
R41	2	DU; duodenal erosions
R12	3	Duodenal erosions; NUD; DU
R14	3	NUD; DU; gastric erosions
R22	3	DU; GU; GU
R6	4	Ca; DU; NUD; NUD
R19	4	DU; RO; GU; GU
R21	5	DU; DU; DU; GU; GU

[a] Laboratory-designated type.
[b] Ca, carcinoma of the stomach; NUD, nonulcer dyspepsia; RO, reflux esophagitis; GU, gastric ulcer; DU, duodenal ulcer.

H. pylori was recovered from different sites within the upper gastrointestinal tract, including the gastric body, antrum, pyloric channel, and first part of the duodenum, in individual patients. The ribotyping patterns of bacteria obtained from these different sites in five patients are shown in Fig. 4. Matching digest patterns for each patient were identical. Another patient, who had two morphologically distinct colonies of *H. pylori* isolated from a single specimen of gastric biopsies, demonstrated identical ribotyping patterns for both isolates, as shown in Fig. 4, lanes 12 and 13.

The ribotyping patterns obtained from *Hin*dIII digestion of *H. pylori* strains isolated from members of seven families are shown in Fig. 5. Ribotyping patterns revealed identical strains among family members in two families (families 1 [father, mother, and son] and 4 [mother and son]). Variability in digest patterns was detected among members of the remaining five families.

DISCUSSION

Several molecular typing techniques have been recently developed for typing *H. pylori*. These are plasmid profile typing and restriction endonuclease analysis of chromosomal DNA (6, 11, 23). These methods, although showing some promise, appear to have some limitations. Plasmid profile typing, which can only be used to type 30 to 40% of *H. pylori* isolates containing plasmids, is also dependent on the stability of the plasmids. Restriction endonuclease analysis of chromosomal DNA has been applied successfully to

TABLE 3. Time intervals between collection of biopsy specimens and ribotyping profiles of *H. pylori* strains isolated from serial follow-up endoscopies of infected subjects

Patient	Time interval (days)	Endoscopic finding[a]	Ribotyping profile[b]
A	0	Prepyloric ulcer	R68
	14	Postoperative pyloroplasty	R68
B	0	Pyloric channel ulcer (DU)	R69
	49	Ulcer healed	R69
C	0	GU	R21
	60	GU healed	R21
D	0	GU	R19
	56	Ulcer unhealed	R19
	133	Chronic GU	R19
E	0	DU and GU	R71
	28	DU persists	R72
	144	DU persists	R71
F	0	Multiple DU	R21
	21	DU healed	R21
	106	Recurrent DU	R21
G	0	DU	R70
	28	DU healing	R70
	119	Ulcer healed	R70
	217	Recurrent ulcer	R70
H	0	GU	R6
	300	Recurrent GU	R6
I	0	DU	R13
	218	DU healed/duodenal erosions	R13
J	0	Duodenal erosions	R74
	41	Duodenal erosions	R74
K	0	DU	R73
	92	DU persists	R73
L	0	Duodenal erosions	R75
	91	Duodenal erosions	R75
M	0	GU	R67
	28	GU healed	R67
N	0	DU healed	R12
	13	Recurrent DU	R12
	148	Recurrent DU	R12
O	0	DU	R76
	122	DU healed	R76
	217	DU healed	R76

[a] GU, gastric ulcer; DU, duodenal ulcer.
[b] Laboratory-designated type.

differentiate strains of *H. pylori* and has been found to be specific and reproducible. This method is useful if applied to a small number of strains for comparative studies, but comparing electrophoretic patterns consisting of up to 1,000 bands is difficult for a large number of strains without the help of expensive, sophisticated scanning devices incorporating pattern-matching software which is not readily available. In the present study, we have found that ribotyping, which produced 2 to 10 DNA bands of digest patterns, was more convenient and practical for a comparative study of a large sample of *H. pylori* isolates.

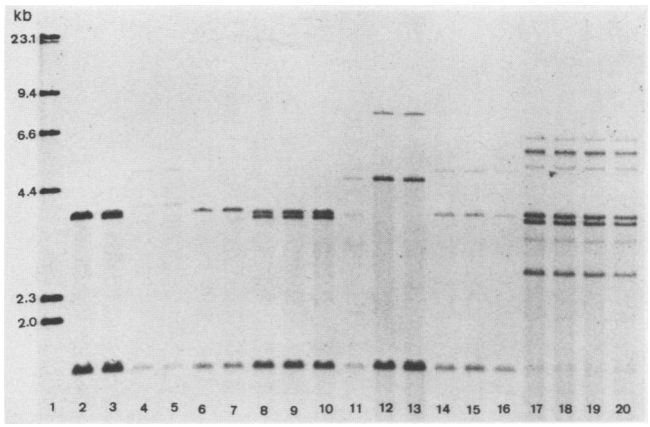

FIG. 3. rRNA gene restriction patterns of *Hin*dIII digests of *H. pylori* DNA in follow-up isolates from infected subjects. Lane 1, molecular weight markers. Multiple isolates are shown in lanes 2 to 20. Lanes 2 and 3, patient A; lanes 4 and 5, patient B; lanes 6 and 7, patient C; lanes 8 to 10, patient D; lanes 11 to 13, patient E; lanes 14 to 16, patient F; lanes 17 to 20, patient G.

The use of rRNA gene restriction patterns as potential taxonomic tools was first proposed by Grimont and Grimont (8). Adopting the same approach, many workers have successfully used this ribotyping method to differentiate organisms such as *Salmonella typhi* (1), *Mycoplasma* spp. (25), and coagulase-negative staphylococci (3). Recently, Morgan and Owen (18) applied this method to study a small sample

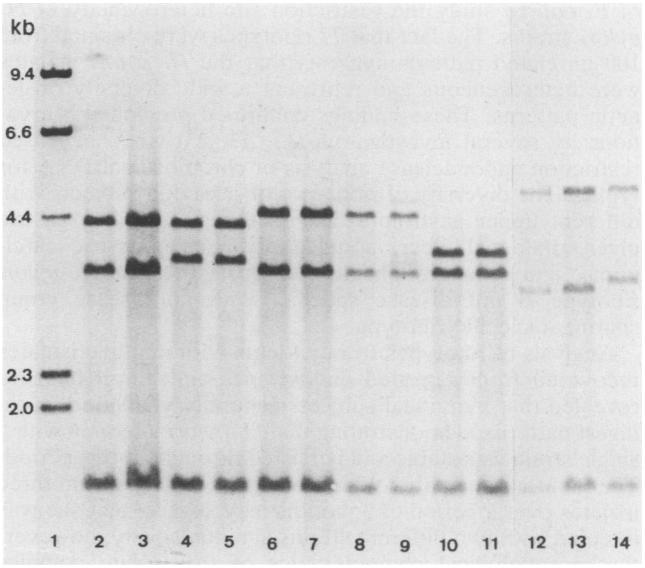

FIG. 4. rRNA gene restriction patterns for *Hin*dIII digests of DNA from *H. pylori* isolates recovered from different anatomical sites within the stomach. Lane 1, molecular weight markers; lanes 2 and 3, patient H (antrum and gastric body); lanes 4 and 5, patient I (antrum and pyloric channel); lanes 6 and 7, patient J (antrum and gastric body); lanes 8 and 9, patient K (antrum and duodenum); lanes 10 and 11, patient L (antrum and pyloric channel); lanes 12 and 13, two morphologically distinct colonies isolated from a biopsy specimen from patient M; lane 14, type strain *H. pylori* NCTC 11637.

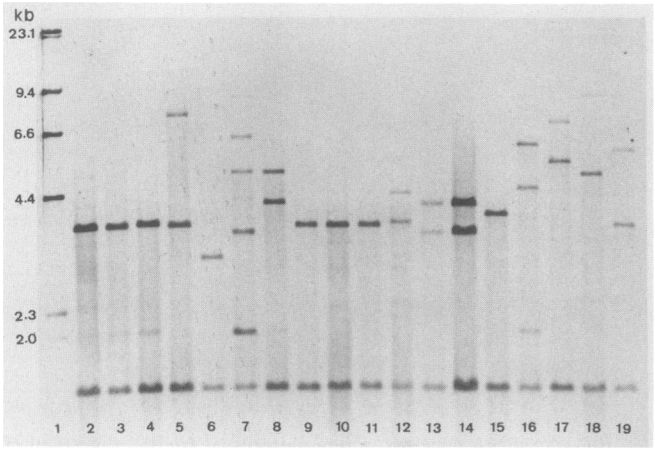

FIG. 5. rRNA gene restriction patterns for *Hin*dIII digests of *H. pylori* DNA from family groups. Lane 1, molecular weight markers; lanes 2 to 4, family 1 (son, father, and mother); lanes 5 and 6, family 2 (son and father); lanes 7 and 8, family 3 (grandson and grandfather); lanes 9 and 10, family 4 (son and mother); lanes 11 and 12, family 5 (brothers); lanes 13 and 14, multiple isolates from patient H; lanes 15 and 16, family 6 (brothers); lanes 17 and 18, family 7 (daughter and mother); lane 19, *H. pylori* NCTC 11637.

(30 cultures) of *H. pylori* using a biotinylated *Escherichia coli* 16S plus 23S rRNA probe. Our results, which encompass a larger sample of clinical isolates, support and expand considerably their findings, even though a slightly different plasmid probe and nonradioactive digoxigenin system was used.

The present study utilized the plasmid pKK 3535 probe which encodes 5S, 16S, and 23S RNA and $tRNA_2^{Glu}$ genes of *E. coli* to study the restriction site heterogeneity of *H. pylori* strains. The fact that 77 ribotypes were obtained from 100 unrelated patients suggests that the *H. pylori* isolates were heterogeneous and represent a wide diversity of genetic patterns. These findings confirmed previous observations by several investigators (11, 13, 23) who have used restriction endonuclease analysis of chromosomal DNA for typing. The diversity of ribotypes observed in subjects with different upper gastrointestinal diseases, including gastric ulcer, duodenal ulcer, nonulcer dyspepsia, gastric carcinoma, and reflux esophagitis, suggests that the *H. pylori* ribotype is not disease specific, with no disease group sharing a specific ribotype.

Analysis of ribotypes from patients with multiple isolates recovered from repeated endoscopies and antral biopsies revealed that individual subjects generally yielded identical digest patterns, demonstrating that *H. pylori* infection with a single strain was stable and persisted throughout this period. The fact that one patient had two digest patterns from three isolates over a period of 3 months may suggest that she was infected with two different strains simultaneously; however, the morphological characteristics of the isolate colonies were not sufficiently distinct during culture to be selected as two different strains for typing. Alternatively, she may have been infected by a different strain after the initial endoscopy. The first strain could still be present but missed during subsequent culture. Langbenberg et al. (11) and Simor et al. (23) found that the same single strain of *H. pylori* persists for consecutive isolates over a period of time. In contrast, Majewski et al. (13) found that consecutive isolates of *H. pylori* were different for 5 of 10 patients. Identical ribotyping profiles obtained from *H. pylori* strains isolated from different anatomical sites of the stomach adds weight to the suggestion of infection by a single strain. Variability of colony appearance within one culture of *H. pylori* has been previously observed (11). Our results with only one culture consisting of two morphologically distinct colonies demonstrated identical ribotyping profiles, confirming the findings of Langenberg et al. (11) that these isolates are genetically identical strains even though they exhibit different colonial morphology.

The mode(s) of transmission of *H. pylori* is yet to be determined. Current evidence provided by the high prevalence of *H. pylori* antibody status of family members of an infected individual (5, 16) and in gastroenterologists (17) would suggest human-to-human spread via fecal-oral and/or oral-oral transmission. More-precise studies by typing strains isolated from family members have been provided by several investigators recently using DNA restriction endonuclease analysis; however, the sample population was usually small (13, 22, 23). Rauws et al. have shown that eight members of one family were infected by a single strain of *H. pylori*. In contrast, two other investigators (13, 23) have reported infection by different strains of *H. pylori* among family members. Our study with seven families suggests that although familial transmission occurs, family members may also frequently acquire infection from different sources. The reservoir of this organism is yet to be found, but the human gastric mucosa is the most likely source.

This study demonstrates that ribotyping of *H. pylori* provided a useful, reliable, and highly discriminatory typing scheme. This technique can be used for the epidemiological study of this infection and for clinical applications, particularly monitoring treatment regimens in order to differentiate between a reinfection with a new isolate or recrudescence due to the same strain of *H. pylori*.

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